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Equine Herpesvirus-1 Outbreak During a Show-Jumping Competition: A Clinical and Epidemiological Study



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ABSTRACT

A total of 752 horses were involved in the CES Valencia Spring Tour 2021. Due to an equine herpesvirus-1 (EHV-1) outbreak, the competition was cancelled and the site was locked down. The objective of this study was to describe epidemiological, clinical, diagnostic, and outcome data of the 160 horses remaining in Valencia. Clinical and quantitative polymerase chain reaction (qPCR) data were analysed for 60 horses in a retrospective case-control observational study. The risk of developing clinical manifestations was explored using a logistic regression approach. EHV-1 was detected by qPCR, genotyped as A2254 (ORF30) and isolated on cell culture. From the 60 horses, 50 (83.3%) showed fever, 30 horses (50%) showed no further signs and 20 (40%) showed neurological signs, with eight horses (16%) hospitalised, of which two died (3%). Stallions and geldings were six times more likely to develop EHV-1 infection compared to mares. Horses older than 9 years, or housed in the middle of the tent were more likely to develop EHV-1 myeloencephalopathy (EHM). These data show that for EHV-1 infection, the risk factor was male sex. For EHM the risk factors were age > 9-year old and location in the middle of the tent. These data highlight the crucial role of stable design, position, and ventilation in EHV-outbreaks. It also showed that PCR testing of the horses was important to manage the quarantine.

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1. Introduction

Equine herpesvirus-1 (EHV-1) is an important, ubiquitous equine viral pathogen that manifests as respiratory disease in young horses, ocular diseases, abortions in pregnant mares, early neonatal death in foals, and myeloencephalopathy (EHM) [1]. Although EHV-1 myeloencephalopathy (EHM) is a relatively uncommon manifestation of EHV-1 infection, it can cause serious losses and severely impact the equine industry, as exemplified by reported outbreaks at riding schools, racetracks, horse shows, and veterinary hospitals throughout North America, Europe, and New

Zealand [2–6]. These different outbreaks have been clinically and epidemiologically described [3–8]. Factors influencing the onset of infection are the viral load, the local immunity in the respiratory tract, and the application of biosecurity measures [3,6]. The viral load is linked to the efficacy of biosecurity measures [8]. Development of viremia may also be influenced by strain differences [9]. Previously, the ability of the virus to cause EHM was thought to be associated with specific EHV-1 “neuropathogenic strains,” which presented a specific DNA polymerase gene mutation (gene open reading frame 30: G2254/N752). Nevertheless, virus strains without this mutation have been isolated in several outbreaks with horses demonstrating neurological signs [7,10–16]. Other known risk factors for EHM include immunity, age, breed, season, and the duration of viremia [3,7,9,17]. The diagnostic tool commonly used in the field for EHV-1 detection is quantitative polymerase chain reaction (qPCR) on nasopharyngeal swabs and/or venous blood col-

Animal welfare/ethical statement: These horses were in real field conditions in a show jumping. There was no ethical animal research file.

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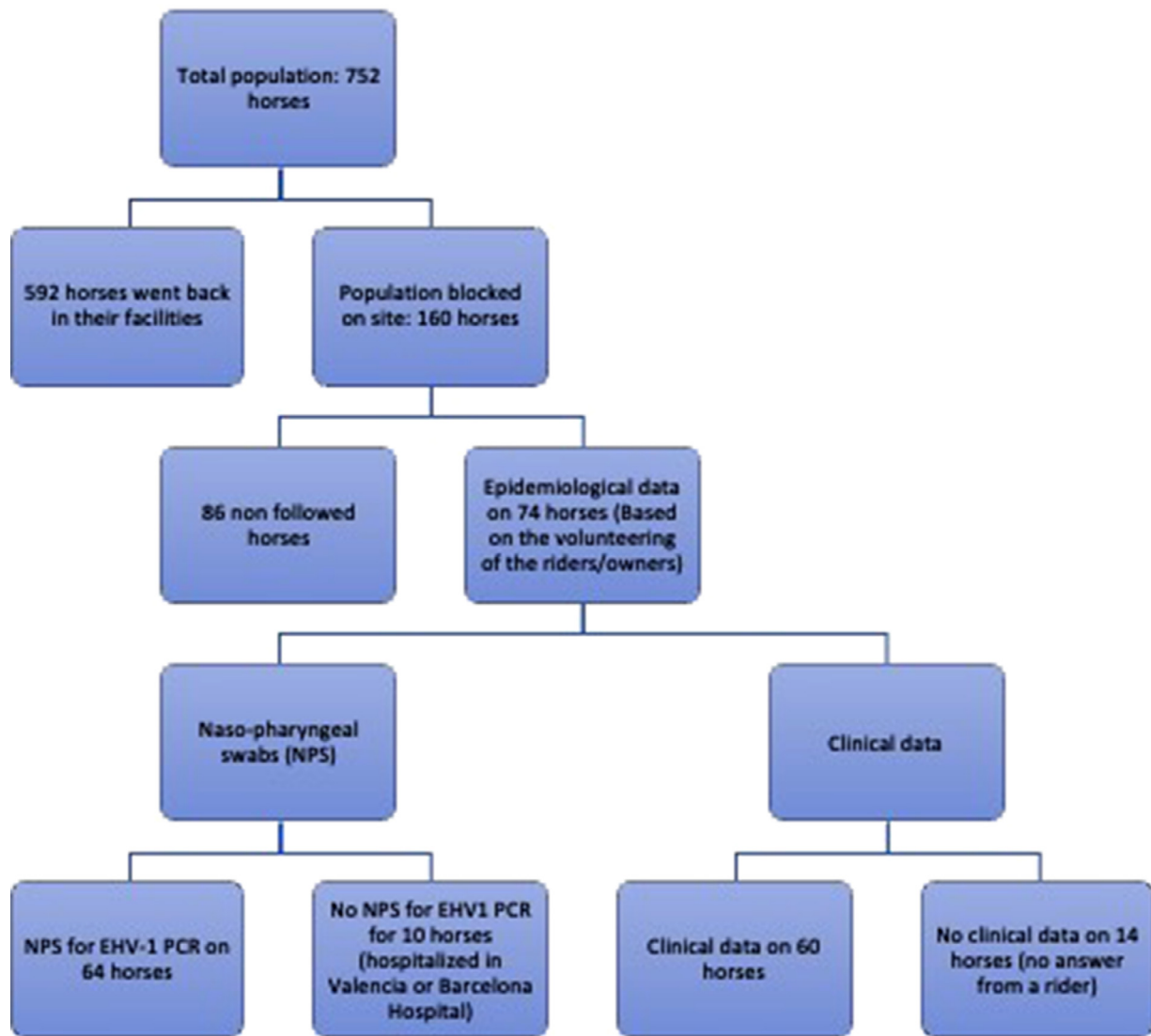


Fig. 1. Diagram workflow presenting the selection of the studied horse population.

lected in EDTA during viremia. This test has a high sensitivity and specificity, is cost-effective, and provides results quickly [18].

This study reports the investigation of an EHV-1 outbreak during the CES Valencia Spring Tour 2021 which was held from the January 28 to the February 21, 2021 in Spain [19]. Sixty horses were monitored during the outbreak and for up to 3 months, for epidemiological data, clinical signs, and qPCR results of EHV-1 in blood and nasal secretions.

2. Material and Methods

2.1. Horses and Premises

CES Valencia Spring Tour 2021 in Spain housed a total of 752 jumping horses during four consecutive weeks. There were between four and nine competitions per day with up to 50 horses per test with one indoor and two outdoor arenas (Fig. 2). A total of 440 temporary boxes (3 × 3m), were installed under a big tent (Figs. 2 and 3). The big tent with the boxes (named E7 on Fig. 2) was constituted of four rows of 20 boxes and 12 rows of 30 boxes with 10 boxes in each block (Fig. 4). Also, there were 3.5 m between each row. There was no ventilation under this tent except for the opening of the tent on the sides (Fig. 5).

On February 20, 2021, the FEI Veterinary Department was informed of horses with pyrexia at the CES Valencia Spring Tour and of a confirmed case of EHV-1 neurological disease in a horse that had recently returned home to France following participation in the event [19]. The tour was subsequently canceled and the site was locked down on February 22 as EHV-1 is a regulated disease controlled by the competent authority with mandatory declaration in Spain. A total of 160 horses remained on site. Among these horses on site, epidemiological, clinical, and diagnostic data recorded either by the riders, owners or veterinarians were available for 74 horses. All owners or caretakers of the horses volunteered to enter this study and provided informed consent that the data from their horses was to be used for a scientific study.

There was no examination of the horses on arrival (no clinical examination with no rectal temperature measurement) and no recommendation to measure rectal temperature once or twice a day during the competition, despite it being specified in the FEI Veterinary Regulations [20]. There was no traffic flow requirement such that horses routinely crossed past each other. There were no facilities for individuals to wash and/or disinfect their hands. A number of dogs were running freely outside and inside the tent and entering the horses' boxes.



Fig. 2. Map of the site of CES Valencia Spring Tour 2021. Source: <https://equipe-online.s3.amazonaws.com/assets/216986/original.png>.

2.2. Horse Movement

Seven horses from England were moved on the February 26 from the tent (E7) to a stable at 10 km from the competition site. On the February 27, about 60 horses without clinical signs were moved from the tent (E7) to permanent boxes on site (Fig. 1, E2). However, these horses were not sampled and tested before being moved. Also, four horses from France were moved on the third of March from the tent (E7) to isolated boxes installed on a parking zone on the competition site (Fig. 1, orange rectangle behind E6).

In order to take French horses back to France, French authorities established and submitted a biosecurity protocol to the Spanish government. The protocol required that it was necessary to have three negative PCR tests 1 week apart to be able to conclude the quarantine period and return home. From the March 11, French horses were taken back directly from Spain to France, with an isolation period at facilities in Lamotte-Beuvron (French Equestrian Federation). The criterion for transportation of the French horses back to France was a thorough clinical exam ensuring horses had no fever, no cough, and no ataxia. The first shipment of horses was undertaken on the March 11 with 10 horses, then on the March 18 with 11 horses, and, finally, on the March 25 with two horses.

2.3. Population Studied

The total population involved in the CES Valencia Spring Tour 2021 represents 752 horses. From these horses, 160 remained on site after the lock down of the site. From these horses, based on the voluntary participation of the owners or caretakers, 74 horses were followed to collect epidemiological data. From these horses 64 (86.5%) were sampled with nasopharyngeal swabbing for EHV-1 PCR as some were not on site (hospitalized in external clinics in Barcelona or Valencia). Sixty horses (81%) had known clinical data. These different populations are shown in Fig. 1. The present study

focused on the 60 horses with complete epidemiological, clinical and laboratory data.

2.4. Collection of Clinical and Epidemiological Data

Clinical data such as HR, RR, rectal temperature, ataxia, lack of tail tone were collected by the Spanish veterinarians on site twice a day during all the period where the horses were on the site. The grade of ataxia was described according to Mayhew's grading system from zero to five [21]. All the horses were examined each day. This included asymptomatic horses.

Details concerning vaccination were gathered directly from the horses' FEI passports but also from data collected by the Spanish veterinarians on site.

2.5. Diagnostic Testing

Nasopharyngeal swabbing of the 64 horses included 21 French horses (32.8%) sampled on the March 3 and 43 horses (67.2%) from six other nationalities March 06, 2021. The swabs were placed in saline 0.9% and sent by FedEx to LABÉO (France) for EHV-1 PCR analysis and virus isolation. On the March 3, the 21 French horses were sampled twice: once with nasopharyngeal swabs (40 cm length) by a French veterinarian who was on site and sent samples by FedEx to LABÉO (France) and once with nasal swabs (12 cm length) by state Spanish veterinarians. Also, official Spanish veterinarians took blood preserved with EDTA for PCR testing. All these samples were sent to a laboratory in Madrid (Spain).

A weekly follow-up of the French horses ($n = 21$) was performed using nasopharyngeal swabs subjected to PCR tests (LABÉO, France). This follow-up was performed for a minimum of 3 weeks and a maximum of 12 weeks according to the results of the horses (positive or negative).



Fig. 3. Position of the boxes inside the tent. There was about 3.5m between two rows of boxes. Photo Anne Couroucé.

LABÉO PCR diagnosis was performed as described by Sutton et al [22]. Briefly, nucleic acids from nasal swabs were extracted using QIAamp Viral RNA Mini Kit extraction kits (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A Real-Time Polymerase Chain Reaction (PCR) was performed on purified nucleic acids for EHV-1 glycoprotein B (gB) gene detection using Diallo et al [23] primers and probe. TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) was used for this PCR. Sensitivity of the PCR test is 108 copies/ μ L of nucleic acid extract. Specificity is 100% after amplification of different EHV-1 (EHV-2, EHV-3, EHV-4, EHV-5, and EHV-9) and other respiratory viruses (EAV, EIV) and bacteria. A second real-time PCR was performed using Sutton et al [22] primers and probes for the ORF30 A/G/C2254 typing. TaqMan Universal Master Mix was also used for this PCR. Results were retrieved as qualitative, with presence or absence of detection. Multi Locus Sequence Typing (MLST) analysis was performed as described by Sutton et al [22].

Virus isolation was performed from nasopharyngeal swab specimens. Nasopharyngeal swabs were collected into a 4 mL sample tube, from which 500 μ L was inoculated on RK13 cells and cultured for up to 12 days (two passages).

2.6. Case Definitions

Horses were classified as controls if they showed no fever and no clinical signs, as EHV-1 cases if they showed fever and as EHM cases if they showed fever and neurological signs. As the competition site was locked because of a confirmed EHV-1 outbreak on site, all horses showing fever were considered as EHV-1 cases. Therefore, PCR results were not included in this case definition.

2.7. Statistical Analysis

Horse characteristics and clinical manifestations were described in terms of a frequency distribution, for categorical data, or as the median, interquartile range (IQR), minimum and maximum values for quantitative data (horse age), because of the non-normal distribution of this variable (Shapiro-Wilk normality test, $P = .006$). Horse characteristics, origin, and vaccination status were compared between the study population, the population on site in Valencia, and the total population of the show to assess the representativeness of our results using the χ^2 test or Fisher's test for qualitative data (horse sex, country of origin and vaccination status) or Kruskal-Wallis test for quantitative data (horse age).

A three-ordered category outcome was considered, where an animal was classified as (1) control if showing no fever and no clinical signs, (2) EHV-1 case if showing fever and (3) EHM case if showing fever and neurological signs. Factors investigated to be potentially separately associated with this outcome included location of the horses (inside, on the edges or outside the tent), sex, age, and duration of the febrile period. Vaccination status effect could be explored only for EHV-1 and EHM cases, as only one horse in the control group was vaccinated, preventing any robust inference. These potential risk factors were investigated using two steps, carried out separately for each potential risk factor. Due to the low number of observations, only univariable analyses were conducted. First, a univariable multinomial logistic regression was carried out to compare (1) controls versus EHV-1 cases and (2) controls versus EHM cases. Second, an univariable ordinal adjacent-category logistic regression was carried out to compare (1) controls versus EHV-1 cases and (2) EHV-1 cases versus EHM cases. Continuous variables (i.e., horse age and duration of the febrile period) were categorized according to their quartile values to explore the shape of their relationship with the outcome variable. When the linearity assumption was violated, their best-fitting form was determined by merging logical categories or categories that reflected changes in regression estimates. For categorical variables with low observations in some cells, the categories were collapsed when biologically relevant. The exponentiated regression coefficients produced risk ratios with each relative risk describing the probability of the outcome in the category of interest relative to the baseline category. These relative risk ratios were referred to as odds ratios (OR). Variables associated to the outcome with a P -value $\leq .05$ were considered significant.

PCR results on nasal swabs collected from the French horses were compared between French and Spanish methods, and between sample types for the Spanish method. The agreement percentage between results was calculated and Kappa coefficient was computed, using the interpretation of Landis and Koch [24].

3. Results

3.1. Horses

For the population of 60 horses, the median age was 9-year old (IQR = 3.25 yo; 6–15 yo). The median age was similar for the 160 horses on site (IQR = 4.5 yo; 5–19 yo) and for the total population of 752 horses (IQR = 4 yo; 5–19 yo). No significant difference was

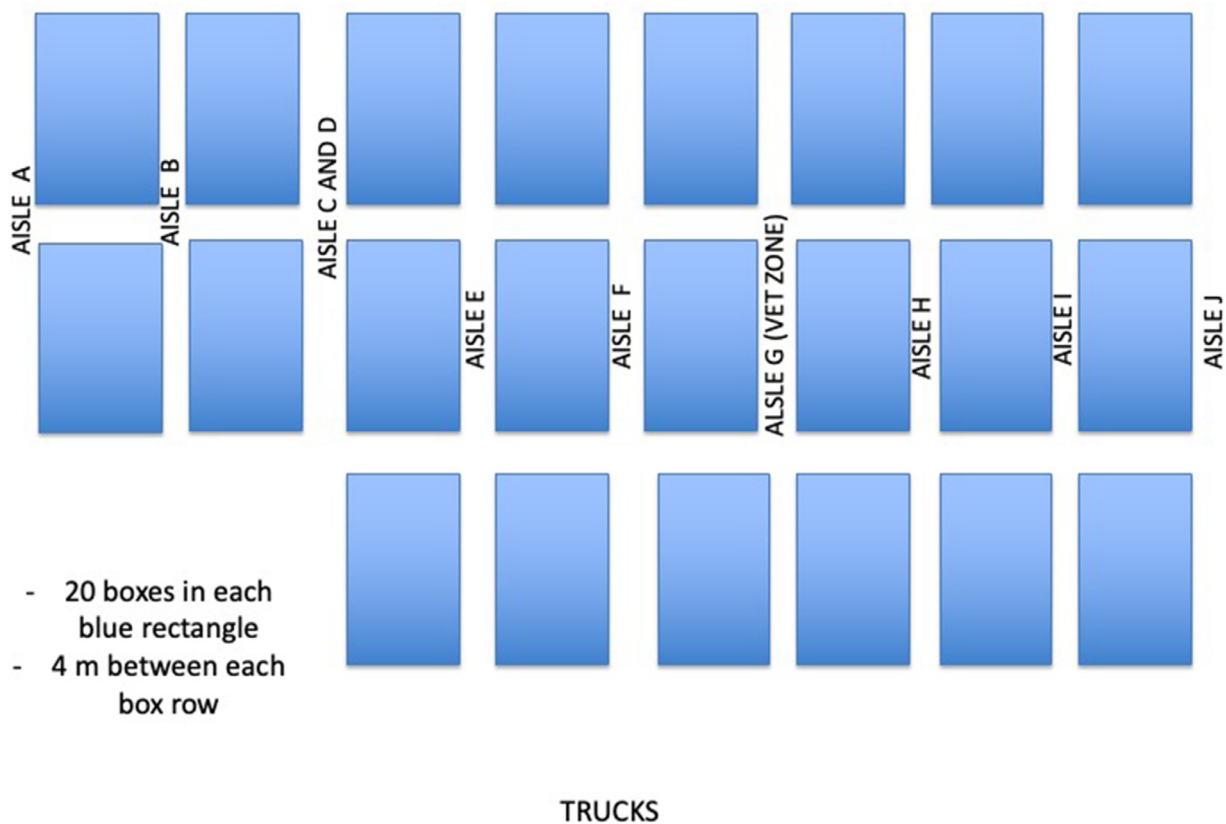


Fig. 4. Positioning of the 440 boxes under the tent. Photo Anne Couroucé.

Table 1

Comparison of the sex, country of origin and vaccination status of the population studied (n = 60 horses), the population on site during the outbreak (n = 160 horses) and the total population of the show (n = 752 horses).

Characteristic	Studied Population (n = 60)	Population Locked on site (n = 160)	Total Population (n = 752)
Sex			
Mare	32 (53.3%)	75 (46.8%)	314 (41.8%)
Gelding	24 (40.0%)	61 (38.1%)	305 (40.6%)
Stallion	4 (6.7%)	24 (15.1%)	133 (17.6%)
Vaccination status			
Vaccinated	33 (55%)	43 (26.9%)	0
Unvaccinated	27 (45%)	114 (71.2%)	0
Unknown	0	3 (1.9%)	752
Country of origin			
France	22 (36.7%)	22 (13.8%)	235 (31.2%)
Canada	3 (5%)	3 (1.9%)	13 (1.7%)
Denmark	4 (6.7%)	12 (7.6%)	35 (4.8%)
Belgium	13 (21.7%)	17 (10.7%)	100 (13.3%)
England	7 (11.7%)	12 (7.6%)	13 (1.7%)
Netherlands	6 (10%)	12 (7.6%)	25 (3.3%)
Germany	5 (8.3%)	17 (10.7%)	63 (8.4%)

observed between our studied population, the population locked on site, and the total population (Kruskal-Wallis test, $P = .93$).

The population (n = 60) comprised 32 mares (53.3%), 24 geldings (40%) and four stallions (6.7%). There was a total of 22 horses from France (36.7%) from three different stables, three from Canada (5%), four from Denmark (6.7%), 13 from Belgium (21.7%) from two different stables, seven from England (11.7%) from two different stables, six from The Netherlands (10%) and five from Germany (8.3%) from two different stables. The comparison for both country of origin and sex with the population on-site and the total population of horses is shown in Table 1. No significant difference was observed in the proportions of horse sex between our studied population, the population locked on site, and the total population (X^2 test, $P = .15$). There was a significant difference between

the proportions of the horse country of origin between our studied population, the population locked on site, and the total population (Fisher test, $P = .0005$). However, there was no significant difference between our studied population and the population on site (n=160; $P = .34$).

With respect to vaccination status, complete information was known for 60 horses. Of these horses, 27 were not vaccinated for EHV-1 (45%) and 33 were vaccinated (55%). Thirty-one horses were vaccinated every 6 months and two were boosted at intervals of 7 or 8 months. The mean duration between the last vaccination and the start of the competition was 1.42 ± 1.59 months (median = 1.0; 0–5 months). The vaccine used for all horses during the previous years was EQUIP EHV 1,4 ZOETIS. Prior to 2017 some horses were administered: Pneumabort-K EHV1 (ZOETIS US), Du-



Fig. 5. Opening of the tent for ventilation. Photo Anne Couroucé.

vaxyn EHV 1,4 (ZOETIS), Pneumequine EHV1 (MERIAL), Prevaccinon EHV1 (INTERVET Deutschland), Bioequin H (IPVET). Vaccination began in 2011 for the older horses and in August 2020 for the youngest. Considering the on-site population of 160 horses, 43 (26.9%) were vaccinated, and 114 (71.2%) were not vaccinated with information not available for three horses (1.9%). There was a significant difference between our studied population (more horses vaccinated compared to nonvaccinated) and population on site (more horses nonvaccinated compared to vaccinated) ($P < .0001$; Table 1).

3.2. Clinical Signs

Of the 60 horses, 10 (16.7%) were classified as control horses for further analysis (Table 2). Of these 10 horses, seven were English horses that were moved from the competition site on the February 26, to a stable which was 10 km from the competition site. For horses showing fever ($n = 50$), rectal temperatures ranged from 38.6 to 41.2°C. Fever lasted between 1 and 10 days with a mean value of 4.0 ± 2.1 days (median = 4.0; 1–10 days). A total of 30 horses (50.0%) showed no further signs and were considered as EHV-1 cases whereas 20 (33.3%) demonstrated additional neu-

rological signs and were considered as EHM cases (Table 2). For these 20 horses, the delay between the last day of fever and the start of neurological signs was from 1 to 4 days (median = 1.0) with a mean value of 1.1 ± 1.3 days. Eight horses (13%) were hospitalized either at the University of Valencia (six horses) or at the university of Barcelona (two horses). Two horses (3.3%) were euthanized and six (10%) were discharged from the hospital. A third horse died after returning home because of severe laminitis. With respect to the neurological signs, these included ataxia (from mild to severe) and/or urinary problems with bladder atony and/or lack of tail tone. None of the 16 horses with neurological signs that remained on site became recumbent. However, of the eight horses hospitalized all became recumbent albeit temporarily. The two that were euthanized were recumbent and unable to rise.

Among the 24 French and Canadian horses that were followed up to 12 weeks, a variety of complications were observed. Six horses showed a second spike of body temperature with five of them showing mild to severe bacterial pneumonia diagnosed by a clinical examination and thoracic ultrasound. Four horses showed mild to severe laminitis and one of them had diarrhea. One horse showed tachycardia and was hospitalized at the School of Veterinary Medicine, Valencia. Measurement of troponin I, echocardiography and electrocardiography revealed myocarditis. This horse also had laminitis. Also, two horses experienced ocular problems with conjunctivitis.

3.3. Vaccination and Clinical Signs

Among the 60 horses, 27 (45%) were not vaccinated and 33 (55%) were vaccinated with 31 being vaccinated according to recommendations (i.e., booster less than 6 months before the show). Among the 31 correctly vaccinated horses, one (3.2%) had no fever and no further signs, 15 (48.4%) showed fever and 15 (48.4%) showed EHM. Of these, three (9.6%) horses died. Both horses that had a booster >6 months previously developed fever. Neither showed neurological signs. Among the 27 unvaccinated horses, nine (33.3%) showed no fever and no further signs, 13 (48.2.7%) showed fever and five (18.5%) showed EHM (27.8%). None of the English horses, which were moved on another site, were vaccinated and none of them showed any clinical signs. In the studied population, vaccinated horses represented 10% of controls, 56.7% of EHV-1 cases and 75% of EHM cases (Table 2).

3.4. Risk Factors

When comparing control horses versus EHV-1 cases, stallions or geldings were six times more likely to be EHV-1 cases compared to mares (P -value = .04; Table 2, OR₁). Age and tent location were associated with development of EHM, compared to control horses (Table 2, OR₂): horses older than 9 years (OR = 6.0, P -value = .05), or housed inside the tent (OR = 9.33, P -value = .016), were more likely to develop EHM compared controls. No significant association of the explored factors was reported on the development of EHM in addition to EHV-1 (Table 2, OR₃). Vaccination status was not associated with the development of EHM in addition to EHV-1 infection (Table 2).

3.5. Diagnostic Testing

Among the 64 horses sampled, there were 29 (45.3%) positive results. Out of the 21 horses tested by both French and Spanish laboratories for nasal sampling, a 57.1% agreement was observed (Table 3A). Kappa's coefficient was 0.22, suggesting a fair agreement between PCR methods. For the Spanish method, an 85.7% agreement was observed between nasal swabs and blood sam-

Table 2
Potential associated variables with development of different clinical signs, 60 sport horses, Valencia, Spain, 2021.

Factors and related data	EHM (N = 20)	EHV-1 (N = 30)	Controls (N = 10)	EHV-1 versus Controls			EHM versus Controls			EHM versus EHV-1		
				OR ₁	95% CI	P-value	OR ₂	95% CI	P-value	OR ₃	95% CI	P-value
Sex												
Stallion or Gelding	8	18	2	6.0	1.08–33.27	.04*	2.67	0.44–15.96	.28	0.44	0.14–1.41	.17
Mare	12	12	8	Ref.			Ref.			Ref.		
Age									.05*			
> 9 y	12	13	2	3.06	0.55–16.90	.20	6.00	1.00–35.91		1.96	0.62–6.19	.25
≤ 9 y	8	17	8	Ref.			Ref.					
Duration of the febrile period (days)	4.7	3.6	0	-	-	-	-	-	-	1.30	0.98–1.79	.08
EHV-1 PCR result on NS	4	13	2	3.06	0.55–16.90	.20	10.0 Ref.	1.44–69.26	.02*	3.27	0.83–12.81	.09
Positive	10	17	8	Ref.						Ref.		
Negative	6	0	0									
Missing value												
Tent location									.016*			
Inside	14	17	2	5.23	0.95–28.91	.06	9.33	1.51–57.65		1.78	0.54–5.91	.34
Outside or on the edges	6	13	8	Ref.			Ref.			Ref.		
EHV-1 vaccination status	15	17	1	-	-	-	-	-	-	2.29	0.66–7.95	.19
Vaccinated	5	13	9							Ref.		
Unvaccinated												
Outbreak outcome												
Dead	3	0	0	-	-	-	-	-	-	-	-	-
Alive	17	30	10									

Univariable multinomial logistic regression to compare control horses versus EHV-1 cases versus EHM (OR₁ and OR₂, respectively) and univariable ordinal adjacent-category logistic regression to compare EHV-1 cases versus control horses, and EHM cases versus EHV-1 cases (OR₁ and OR₃, respectively). With OR, Odd Ratio; CI, Confidence Interval; NS, Nasopharyngeal swab

Table 3A
Comparison of PCR results for nasal swabs in Spain and nasopharyngeal swabs in France.

	Results Spain		Total
	Positive	Negative	
Results France			
Positive	7	8	15
Negative	1	5	6
Total	8	13	21

Table 3B
Comparison of PCR results for nasal swabs and blood samples in Spain.

	Results nasal swab		Total
	Positive	Negative	
Results blood sample			
Positive	6	1	7
Negative	2	12	14
Total	8	13	21

ples (Table 3B). Kappa’s coefficient was 0.69, suggesting substantial agreement between the two sample types.

Follow-up of the French horses (n = 22) showed 16 horses (72.7%) sampled on the March 3 with a positive PCR. Seven horses out of these 16 (43.7%) became negative after 1 week and remained negative thereafter. Of the nine (56.3%) other horses, some became negative after 2 or 3 weeks, some became negative for 2 weeks and then returned to being positive again, with some remaining positive over 56 days (Table 4).

The EHV-1 strains detected during the outbreak were characterized by genotyping, Multilocus Sequence Typing (MLST) and whole genome sequencing. All the strains detected during the outbreak were A2254 (ORF30). Viruses from two horses from France that remained in Valencia were isolated on cell culture. The two strains

were characterized by MLST as belonging to clade 10. These results are detailed in previous publications [25,26].

4. Discussion

This study investigated an EHV-1 outbreak that occurred during a show-jumping event in Valencia. The studied population was representative of the horse population locked on site in terms of age distribution, and proportions of sex and country of origin, supporting extrapolation of the present results to the complete population locked on site. The studied population was also representative of the age distribution and sex proportion of the whole horse population attending the show.

4.1. Clinical Signs

EHM is a serious manifestation of EHV-1 infection that affects the central nervous system. Affected horses show mild to moderate respiratory disease and fever, with 2.2% to 78.3% of infected horses developing EHM which clearly indicates the highly variable presentation of this disease [7,9,27,28]. Fever can be indicative, although it is not specific to EHV-1. Goerhing et al [17] showed in an experimental infection that the fever peak follows the increase in viral load 2 to 3 days after infection. Pronost et al [29] reported that during an EHV-1 outbreak, involving 7 of 66 horses presenting with EHM, there was a strong association between fever and subsequent ataxia observed 2 days after. Henninger et al [3] showed that out of 135 horses at a facility, 117 (86.7%) displayed signs of EHV-1 infection. Forty-six horses (39.3%) developed EHM. These percentages are similar to our results.

In horses with EHM, clinical signs of ataxia and weakness due to spinal cord damage can be mild to severe, asymmetrical or symmetrical, and lead to recumbence (paralysis or tetraplegia) in severe cases [9]. Usually, the pelvic limbs are more severely affected than the thoracic limbs, resulting in more notable hind limb weakness, bladder dysfunction with urine dribbling, sensory deficits in the perineal area, and decreased tail and anal tone with fecal re-

Table 4

Follow-up of the 21 French horses and the three Canadian horses: positive horses (in red) and negative horses (in green) following nasopharyngeal swabs and PCR test at LABÉO Frank Duncombe (Caen, France).

	Vaccination	Fever	Duration between fever and NPS	EHM	03/4	03/9	03/16	03/23	03/30	04/06	04/12	04/19	04/30	05/30
H1	No	No	-	-	N	N	N							
H2	No	Yes	14	NO	N	N	N							
H3	Yes	Yes	16	YES	N	N	N							
H4	No	Yes	18	NO	N	N	N							
H5	Yes	Yes	10	NO	P	P	N	N	N					
H6	Yes	Yes	3	YES	P	N	N	N	N					
H7	Yes	Yes	13	NO	N	N	N	N	N					
H8	Yes	Yes	7	YES	P	N	N	N	N					
H9	Yes	Yes	13	YES	N	N	N	N						
H10	Yes	Yes	12	YES	P	ND	ND	P	P	P	P	P		Dead
H11	Yes	Yes	12	YES	P	P	P	N	N	N				
H12	Yes	Yes	10	NO	P	N	N	N						
H13	No	Yes	11	YES	P	P	N	N	N					
H14	Yes	Yes	12	NO	P	N	N	N						
H15	Yes	Yes	10	NO	P	N	N	N						
H16	Yes	Yes	11	NO	P	P	P	P	P	P	P	P	N	N
H17	Yes	Yes	5	NO	P	P	P	N	N	N				
H18	Yes	Yes	10	NO	P	N	N	P	P	N	P	N	N	N
H19	No	Yes	7	YES	P	N	N	N						
H20	No	Yes	13	NO	P	N	P	P	P	N	N	N		
H21	Yes	Yes	12	NO	P	P	N	N	N					
H22	Yes	Yes	13	YES	P	N	N	N						
H23	No	Yes	10	YES	ND	ND	N	N	N	N				
H24	Yes	Yes	11	YES	ND	ND	ND	N	N	N				

Three negative PCR tests in a row, 7 days apart, was considered as appropriate criteria for the horses to return home.

P, Positive; N, Negative; ND, Not Done

tention occur also [30,31]. This list of clinical signs is similar to what we observed in our population with one difference; none of the horses in our population showed fecal retention.

In the studied population, five horses among the 24 French and Canadian horses showed a second spike of temperature as a result of bacterial pneumonia. Primary infection with viruses such as influenza, for example, is very often complicated by secondary bacterial infection, usually associated with *Streptococcus zooepidemicus* or *Pasteurella/Actinobacillus* [32]. When this occurs, a second febrile response is observed 2 to 3 days after the initial viral-induced pyrexia, invariably higher and of longer duration than the first one [32]. It is associated with a mucoid to mucopurulent nasal discharge, increased coughing and signs of bronchial and lung involvement, which if untreated, will frequently result in bronchopneumonia and pleuritis [32]. These French and Canadian horses also showed complications such as myocarditis and laminitis. To our knowledge, there is no publication showing the link between EHV-1 and myocardial damage. However, in humans, an inflammatory cardiomyopathy is predominantly induced by viral infection [33]. In horses, a study demonstrated no evidence of severe myocardial necrosis secondary to EIV challenge in sedentary ponies. However, transient increases in Troponin I suggest that mild myocardial damage may occur [34]. For laminitis, there may be some relationship to the high dosage and frequent administration of corticosteroids these horses received.

Our study showed that the delay between the last day of fever and the start of neurological signs lasted from 1 to 4 days with a mean value of 1.1 ± 1.3 days. This is consistent with the onset of EHM which typically occurs between 6 and 10 days after the initial EHV-1 infection (1–3 days after resolution of a fever), often suddenly, and reaches peak severity within 2 to 3 days [30].

Our study found that there were three horses that died from the population of 60 horses (5% of the total population of 60 horses and 15% of the horses showing neurological signs) and FEI recorded 20 dead horses (2.6%) among the 752 horses which were in Valencia [19]. This is in accordance with the rate of dead horses reported in Odgen: 10 of 421 (2.4%) [27]. However, it represents

a much lower rate of death than the rates reported in previous studies of EHM: rate of 46% in a retrospective study on 26 EHM cases in France [12] and 26% of EHM cases in the United States representing 8.9% of the entire population [3]. The lower percentage of horses dying in our study could be due to the fact that many of the horses were no longer on site likely leading to decreased exposure to the virus.

4.2. Risk Factors

Although stallions and geldings were six times more likely to develop EHV-1 infection when compared to mares, there was no significant effect of sex on the development of EHM. These results are consistent with those of Henninger et al [3] but differ from others who found that mares were more likely to develop EHM compared with controls [2,28]. This latter difference might be due to the small size of our population increasing the risk of a Type II error or failure to detect a difference.

Horses older than 9 years were six times more likely to develop EHM compared to controls. These results are consistent with the previously reported increased risk of neurologic disease related to EHV-1 infection in older horses [3,6]. There is some evidence that in mares over 20 years of age the incidence of clinical EHM increases by up to 70% [35,36].

Horses housed in boxes located in the middle of the tent were over nine times more likely to develop EHM versus controls, when compared to those horses located outside or on the edges of the tent. We postulate that the former group of horses may have been exposed to less air renewal because of the absence of ventilation, leading to greater exposure to virus aerosols shed by sick horses.

Horses with a positive EHV-1 PCR result were 10 times more likely to develop EHM versus control horses, supporting the EHV-1 cause for this outbreak. Several stress factors have been associated with the reactivation of latent EHV-1 in previous studies, such as the long transport of sport horses from their stable to the competition venue, but these could not be assessed here. One study showed that among 302 healthy adult horses with a history of

recent transportation to a quarantine facility, three horses were PCR-positive for EHV-1 in blood or nasopharyngeal secretions [37]. Following reactivation, viruses are delivered to the respiratory epithelium, where virions are either neutralized by local mucosal immunity or replicated leading to an infection characterized by virus shedding in respiratory tract secretions and eventual development of viraemia [37]. However, even if an infection is established, it generally remains asymptomatic and results in silent shedding of the virus. This could be what happened with the unknown index case in Valencia with transport and stress resulting in a reactivation of the virus. However, we cannot exclude the arrival of a horse that was excreting the virus without showing any clinical signs.

4.3. Vaccination

Vaccination against EHV-1 is highly recommended but is not mandatory in sport horses. In France, EHV-1 vaccination is mandatory for racehorses (Trotters and Thoroughbreds) and for young sport horses (Société Hippique Française – SHF) but not for sport horses and the RESPE (Réseau d'Epidémiologie et de Surveillance en Pathologie Equine – French network for the surveillance of equine diseases) estimates that about 30% of the French horse population is vaccinated. In Previous studies have shown a high rate of vaccination with 96% horses vaccinated against EHV-1 in the year preceding the outbreak in one study [3] and 73.3% horses that were vaccinated against EHV-1: 70.9% for control horses, 75% for EHV-1 cases and 83.3% for EHM cases in another [2]. In Valencia, in the total population of international horses on site (752 horses), only 26.9% were vaccinated. In our studied population (n = 60), 55% of the horses were vaccinated: 10% of the control horses, 57% of EHV-1 cases and 75% of EHM cases. The vaccination status was not associated with the probability for a horse to develop further EMH when being infected by EHV-1.

4.4. Laboratory Diagnosis

4.4.1. EHV-1 PCR

PCR detection of EHV-1 is routinely performed on secretions from nasal or nasopharyngeal swabs or uncoagulated blood samples [9]. PCR has become the diagnostic test of choice because of its high sensitivity and specificity [18]. The real-time PCR assay can detect as little as 1 TCID₅₀ of EHV-1 virus and approximately 0.15 pg of DNA with a specificity of 99.2% [23]. In the current study, the follow-up of French horses showed that some horses remained positive for PCR following a nasopharyngeal swab for 56 days. Previous work showed a similar pattern, with the prevalence of EHV-1 infection in nasopharyngeal swabs decreasing from 25% to 14% over the 22 days of the study period [38]. These authors also showed low viral loads in blood and up to 1,000,000-fold higher viral loads in nasal secretions in EHM horses. In addition, they showed that viral loads in the nasal secretions of neurological horses were significantly higher than those of either febrile or subclinically infected horses [38]. These findings suggest the contagiousness of neurologically affected horses. But the viral load measured by PCR associated with myeloencephalopathy cases could be lower due to the later onset of neurological signs compared to respiratory signs and peak fever [29].

It is not clear if the technique of sampling may be a source of different results, observed in this study, between the two diagnostic laboratories. When comparing nasal and nasopharyngeal swabs, one study showed that nasal swabbing represents a viable alternative to the less well tolerated nasopharyngeal swabbing technique for the molecular detection of EHV-1 in horses [39]. However, another study showed that, for EIV detection, nasopharyngeal swabs were superior to nasal swabs, with increased frequency and amount of virus detected [40]. The difference may also come

from PCR techniques used. Sensitivity and specificity of real-time PCR assays can be affected by a variety of factors such as sample type, sample volume, viral nucleic acid extraction method, target gene, primers and probes and their concentrations, commercial PCR reagent kits, number of cycles, and cutoff point [18,23]. These results underline the importance of a good and standardized sampling method and a laboratory with adapted diagnostic tools with internal quality control. In Valencia, PCR testing was not done before moving horses from the tent to the area with permanent boxes. Therefore, from the February 28, up to three horses per day were removed from these permanent boxes and returned to the tent following detection of fever.

PCR results may also help when assessing viral loads in blood and nasal or nasopharyngeal secretions. This can be used as a guide for modifications to control measures, including the lifting of quarantine for individual horses that test negative on subsequent sampling [31]. This was done for the French horses as three negative PCR tests on nasopharyngeal swabs were necessary before the horses were allowed to leave quarantine and return home. Harmonization of real-time PCR protocols between laboratories is very important in order to be able to compare results. The threshold cycle (Ct) values can be used to indicate the approximate viral DNA concentration in samples during a crisis, but we must be conscious that Ct values differ between laboratories for the same sample. Only a quantitative method with international units (i.e., GE copies/mL of sample) would allow comparison between laboratories. However, positive PCR results do not necessarily equate with the risk of an infected horse transmitting infection to a susceptible cohort [41]. Effectively, our results show that 76% (13/17) EHV1 cases and 71% (10/14) EHM cases had a negative EHV-1 PCR result at the time of the sampling at the beginning of March. This highlights how the timing of PCR testing is important for diagnosis.

4.4.2. Virus Isolation and Characterization

Only isolation by cell culture can prove the infectious status of the virus. Characterization is also important (1) to know if the virus is close or not to other viruses circulating in Europe and around the world and (2) to have a traceability of the virus during other outbreaks.

Nucleotide sequence analysis of ORF30 is used to confirm the presence of EHV-1 and characterize the genotype (A2254 referred to as a non-neuropathogenic strain or G2254 a neuropathogenic strain) [42,43]. However, many studies describe the detection of G2254 strains associated with abortion and A2254 associated with EHM [44–46]. This was the case in Valencia: all the strains involved in the outbreak were A2254, which is regarded as non-neuropathogenic strain [42]. The recent discovery in Europe and US of cytosine in position 2,254 (C2254) reinforce the limit of this designation which is overly simplistic [22,47].

A MLST approach allows identification of the different EHV-1 clades described by Bryant et al after whole genome sequencing [13,16,48]. MLST study revealed that the two isolated FR/Valencia1/2021 and FR/Valencia2/2021 (accession number: MW855961 and MW855962, respectively) strains belonging to clade 10, were closely related to other viruses circulating for several years in Europe. Analysis of all the MLST sequence fragments revealed the presence of a mutation in the Valencia EHV-1 virus in ORF11. The A713G mutation detected was not present in any other EHV-1 strains described in data banks and could be used as a tracer to differentiate the Valencia EHV-1 strain from other strains [25].

Whole genome sequencing confirmed that the two French strains but also the three Belgian strains from the Valencia crisis belong to clade 10 [26]. The A713G mutation was identified in these five strains. Later, the whole genome of an EHV-1 strain from Switzerland, isolated from a horse present in Valencia, confirmed

the presence of both SNPs A2254 and G713 in ORF30 and ORF11, respectively [49]. The G713 marker was used to characterize the strains detected in France since the Valencia episode, particularly the abortions. To date, no strain has shown the presence of a G base at position 713 of ORF11.

5. Conclusion

This study features epidemiological, clinical and diagnostic data collected in a real field situation. Relevant findings of this study are the importance of stabling design, position in the stables but also ventilation and horse movements. Biosecurity risks were important in Valencia with a high number of horses in the same tent with no ventilation and no biosecurity measures becoming infected. The occurrence of this outbreak supports recommendations for preventive measures such as controlling the horses on arrival with a clinical examination including measure of rectal temperature before entering the stables and monitoring during the competition as horses may reactivate an EHV-1 infection some days after arrival because of the stress of transport or other factors. It also underlines the importance of having isolation boxes and adequate procedures to be able to immediately isolate horses with fever. Finally, this study showed that testing with PCR is a useful adjunct for biosecurity and quarantine, although there may be different results according to the sampling technique and also the laboratory.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Anne Couroucé: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Formal analysis, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing. **Camille Normand:** Validation, Visualization, Writing – original draft, Writing – review & editing. **Caroline Tessier:** Investigation. **Rita Pomares:** Investigation. **Jérôme Thévenot:** Investigation. **Christel Marcillaud-Pitel:** Investigation. **Loïc Legrand:** Investigation. **Pierre-Hugues Pitel:** Investigation. **Stéphane Pronost:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Coralie Lupo:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Informed consent

All the owners and caretakers of the horses have been informed and accepted that the data from their horses were used for a scientific study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2023.104869.

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